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Abstract Approved:
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Astragalus oniciformis Barneby is a xerophyte of the sagebrush deserts of central Idaho. It is a narrow endemic of the upper Snake River Plains where it inhabits stabilized, aeolian sand deposits over Quaternary basalt flows. The objective of this study was to determine the levels and distribution of genetic differentiation within and among populations of *Astragalus oniciformis*. Fifteen individuals from each of eight populations, chosen from throughout the range of the species, were selected for their accessibility, density of individuals, and large population size. Two disjunct eastern populations selected for this study have been separated from the continuous western populations for 3600 years by an eight-mile wide, inhospitable lava flow. Inter-simple sequence repeats (ISSR) were chosen as the marker to assess genetic differentiation. Two primers were selected that yielded 40 loci, all of which were polymorphic in *A. oniciformis*. In an analysis of molecular variance (AMOVA), 88.69 percent of the variation was significantly attributed to variation within populations. The differentiation between the two disjunct populations and the western populations was insignificant. High gene flow (Nm = 3.91-3.93) and a low percent deviation from Hardy-Weinberg equilibrium due to population subdivision (G_{st} = 0.113-0.1134) were found among populations of *A. oniciformis*. These results suggest that current threats to this species, ranging from plant community changes due to changing fire patterns, habitat alteration from livestock grazing, and habitat loss from agricultural development have not yet

affected the genetic diversity of this species. Preservation of the numerous, large populations and the high gene flow will help insure that the levels of genetic diversity found in *Astragalus oniciformis* will not decrease.

Genetic Diversity of Populations of *Astragalus oniciformis* using Inter-Simple Sequence Repeat (ISSR) Markers

by
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APPROVED

Major Professor, representing Botany and Plant Pathology

Chair of Department of Botany and Plant Pathology

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

J. Andrew Alexander, Author

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The author also thanks Steve Popovich, who selected the populations of *Astragalus oniciformis* that were sampled, lead the field trip to collect the samples, and sampled additional populations not visited on the original field trip. He also provided the population survey data used as background information for this study.

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INTRODUCTION

Astragalus oniciformis Barneby (Fabaceae) was first collected by Ripley and Barneby in 1947 in the foothills of the Sawtooth Range on the eastern edge of the town of Picabo, Idaho (Barneby, 1964). For thirty years, this single site remained the only known occurrence of this species. In the seventies and later in the early eighties, Shoshone District Bureau of Land Management (BLM) employees completed surveys that identified few additional populations. The first comprehensive survey was completed in 1984 by Packard and Smithman (Moseley and Popovich, 1995). Populations of *A. oniciformis* were reinvestigated in 1994 and 1995 by Moseley and Popovich. Their report, *The Conservation Status of Picabo Milkvetch (**Astragalus oniciformis** Barneby),* remains the most comprehensive inventory and natural history study of this species. Thirty-six populations of *A. oniciformis* have now been recorded. Population sizes range from 10 to greater than 10,000 individuals. Twenty-nine of the thirty-six populations from previous studies were located and studied by Moseley and Popovich. The seven remaining populations have not been relocated since 1984 and are considered historical populations. None of these historical populations are known to be extirpated (Moseley and Popovich, 1995).

The populations of *A. oniciformis* are spread throughout Lincoln, northern Minidoka, and southern Blaine Counties, Idaho. However, in the eastern portion of its range, several populations are separated from the central populations by the Minidoka Flow, an inhospitable, eight mile wide, basaltic lava flow. This flow has been dated at 3600 years and is too young for erosional or depositional processes to form suitable habitat for *A. oniciformis*. In the western portion of its range, two populations, located 5.8 to 6 miles west of Shoshone, are historical and their current status is unknown (Moseley and Popovich, 1995).

Astragalus oniciformis is a prostrate, caulescent perennial herb that establishes in disturbed sandy areas or sandy, aeolian pockets on basaltic lava flows (Barneby, 1964). Throughout its range it occurs with *Artemisia tridentata* Nutt. var. *wyomingensis* (Beetle & Young) Welsh and *Hesperostipa comata* (Trin. & Rupr.) Barkworth. It is frequently found within open grassy areas of previously burned patches among *Artemisia* scrubland, rather than in the understory of late seral *Artemisia* stands. *Astragalus oniciformis* prefers stabilized sandy pockets and open areas and has never been found in unstabilized sand dune environments (Moseley and Popovich, 1995). The sandy soils that *A. oniciformis* inhabits overlie an extensive series of basaltic lava flows that have erupted in this region over the past 750,000 years. In the eastern portion of its range, *A. oniciformis*

populations are found in aeolian deposits on and surrounded by basalt flows ranging in age from 3600 to 12000 years. These flows originated in the Crater of the Moon Lava Field (Moseley and Popovich, 1995).

In his monograph of the genus *Astragalus*, Barneby (1964) placed *A. oniciformis* in Section *Miselli,* a group of xerophytic taxa native to the western United States and montane regions of central Mexico. *Astragalus oniciformis* was placed in this section, in part, based on the presence of free stipules (Barneby, 1964), a feature that Barneby frequently used to segregate species of *Astragalus*.

Astragalus mulfordae Jones was also sampled in this study. Closely resembling *A. oniciformis*, *A. mulfordae* has fused stipules. Based on this feature, Barneby placed it into Section *Neonix*. *Astragalus mulfordae* is distributed from southeastern Oregon to southwestern Idaho and is not sympatric with *A. oniciformis*, although it is closely related (Barneby, 1964). *Astragalus mulfordae* was included in this study as an outgroup and to determine the utility of the ISSR process among species in *Astragalus*

Inter-simple sequence repeat (ISSR) markers have recently become widely used in population studies because they have been found to be highly variable, to require less investment in time, money and labor than other methods (Wolfe and Liston, 1998), and to have the ability to be inherited. (Gupta et al., 1994; Tsumura et al., 1996). Random amplified polymorphic

DNA (RAPD) markers, are a related and more widely employed, genetic method. Expression as dominant markers, homology problems related to bands of equal length, weak bands remaining unobserved due to artifacts of DNA visualization methods, and minor deviations in experimental protocols yielding different results are a few of the limitations shared by these two molecular methods (Wolfe and Liston, 1998). ISSRs, however, are more robust than RAPDs due to using longer, anchored primer sequences (Wolfe and Liston, 1998).

Typically ISSRs have been used in studies using cultivated species for producing of genetic linkage maps and determining the relatedness of lines of agriculturally important species. In producing genetic linkage maps, ISSRs have limited application. Nine of forty-nine ISSR fragments produced by 33 primers were successfully mapped on a restriction fragment length polymorphism (RFLP) linkage map of wheat. These fragments were shown to be distributed over five different chromosomes (Kojima et al., 1998). ISSRs have been more widely used in determining the relatedness and variability of lines of cultivars or finding the most closely related native plant species. For example, in *Sorghum bicolor* ssp. *bicolor* (Poaceae), 81 lines representing all five races were analyzed using RFLP, RAPD and ISSR primers. Four ISSR primers produced 49 markers with a range of eight to nineteen polymorphic

bands. ISSR markers alone were able to distinguish all 81 lines (de Oliveira et al., 1996).

In the cultivated species, *Pandorea pandoreana* and *Pandorea jasminoides* (Bignoniaceae), ISSRs were used to determine relationships of 13 cultivars. Six ISSR primers yielded 112 polymorphic bands from a total of 118. These data combined with 395 bands from a RAPD analysis were used to produce a genetic distance dendrogram of the 13 cultivars. The cultivars were separable into two groups (genetic distance = 0.7) that represented cultivars of *P. jasminoides*, a species endemic to eastern Australia, and *P. pandoreana*, a species that ranges from northeastern Australia to New Guinea (Jain et al., 1999).

ISSRs have also been used to determine the genetic diversity and origin of the last surviving individuals of *Sophora toromiro* (Fabaceae), a tree endemic to Rapa Nui (Easter Island) that has been extinct in the wild since 1960. Forty-three samples of *S. toromiro* and eleven samples of other related species of *Sophora* were taken from live trees in botanical gardens and private collections from throughout the world. Three ISSR primers produced 24 bands. Fifteen of those bands were present in *S. toromiro*. Twenty-one bands were present in the other *Sophora* species, of which 12 were also present in *S. toromiro*. In general ISSR markers in this species showed a lower level of resolution than RAPD data. Two trees thought to be

S. toromiro were conclusively identified as *S. microphylla* with these molecular data, eliminating these two trees as possible sources for seed for reintroduction purposes (Maunder et al., 1999).

In other studies, ISSRs have been successful in distinguishing between subspecies of *Plantago major* (Plantaginaceae), a cosmopolitan species (Wolff and Morgan-Richards, 1998), in determining the closest native species related to the hexaploid *Ipomoea batatas* (Convolvulaceae; sweet potato; Huang and Sun, 2000), and in determining the levels of genetic variation between sympatric species of *Alnus* (Betulaceae) in Italy (King and Ferris, 2000).

ISSRs have also been instrumental in determining variability and correcting misidentifications in large germplasm collections (Fang et al., 1997; Gilbert et al., 1999; Lanham and Brennan, 1999; Charters and Wikinson, 2000).

Several population-level studies have also used ISSR markers. In an octoploid clonal species native to Ohio, *Calamagrostis porteri* ssp. *insperata* (Poaceae), three ISSR primers produced 67 bands. Polymorphic loci ranged from 10.4% to 20.7%. Similarity among populations ranged from 0.712 to 0.757 (Esselman et al., 1999). In contrast, an analysis of *Saxifraga rivularis* (Saxifragaceae) from Great Britain yielded few polymorphic bands. Four

primers were used and only one was polymorphic. An additional band was represented only in collections from Iceland (Hollingsworth et al., 1998).

Differences in levels of polymorphism can exist between ISSR and allozyme data (Esselman et al., 1999), between ISSR data and cpDNA restriction site analyses (King and Ferris, 2000), between ISSR and RAPD data (Jain et al., 1999), and between AFLP and ISSR data (Arcade et al., 2000). These can result in different estimates of diversity within and among populations and species. ISSRs can generate higher percentages of polymorphic loci than other methods (Esselman et al., 1999). In comparative studies, ISSRs have been shown to be as reliable and be as genetically informative as RFLP analyses (Nagaoka and Ogihara, 1997; Huang and Sun, 2000).

Based on the low cost, the ease of experimental replicability, and the robust nature of the method compared to others, ISSR markers were selected to determine the levels and distribution of genetic differentiation among populations of *Astragalus oniciformis*.

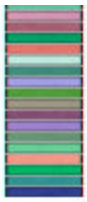
MATERIALS AND METHODS

From the 36 known populations of *A. oniciformis*, populations from throughout the range of the species were selected for sampling. Eight populations were sampled for a total of four population pairs (Figure 1 and Figure 2; Table 1). Population inventory data were based that found in *The Conservation Status of Picabo Milkvetch (**Astragalus oniciformis** Barneby)*. The population pairs ranged from 5 to 16 km apart. An initial collecting survey (J. Alexander, A. Liston, S. Popovich) was conducted in the late spring of 1999. From the northern portion of the range, populations from Ditch Spring (DS) and Silver Creek (SC) were sampled. From the central portion of the range, a population near Lower Thumb Reservoir (LTR) and a population within the Wilderness Study Area III exclosure near Squaw Butte (SB) were sampled. A population (SD) from the southern portion of the range, located in sand dunes 7.5 miles east of the town of Dietrich, was sampled. Steve Popovich collected the remaining samples: a population located southwest of Crater Butte (CBS), and the rift populations, Great Rift #2 (GR2) and Mule Butte (MB) (Moseley and Popovich, 1995).

A population of *Astragalus mulfordae* in Malheur County, Oregon was also sampled during the original survey (Table 2).

Table 3. Population description of the sampled population of *Astragalus diaphanus* from Madheur County, Oregon. Coordinate data was recorded in the field with a commercial GPS unit.

Abbrev.	Location	Lat.	Long.	Prop. river
SH-09	0.9 miles south of Beulvely Hot Springs picnic area	43.7189	-117.1924	Snake



dune sand
felsic pyroclastic
felsic volcanic flow
glacial drift
granite
granitic gneiss
interlayered meta-sedimentary
lake sediment and playa
loess
mafic gneiss
mafic volcanic flow
mixed carbonate and shale
mixed eugeosynclinal
mixed miogeosynclinal
open water
sandstone
siltstone

The Blow Out Southwest
The Blow Out West
Tumupa
Tumupa South
Twin Lakes
Wagon Butte
Way East of Richfield
Cities
County Boundary

Fifteen individuals from each population, for a total of 135 samples, were used for ISSR analyses. Between 20 and 80 mg of leaf material was collected from each individual. Leaf samples were air dried and then stored at -20°C until the DNA was extracted. Genomic DNA was extracted and purified using the DNeasy Plant Mini Kit (QIAGEN, Chatsworth, CA).

17 µl of reactants were used to complete all ISSR reactions. An initial 8.5 µl buffer solution was created using 1.85 µl of ddH_20, 1.02 µl of 25 mM $MgCl_2$ (Promega, Madison, WI), 0.53 µl of an equal mixture of 2.5 mM dATP, dCTP, dGTP, and dTTP (Epicentre, Madison, WI), 1.7 µl of 10X buffer (Promega), 3.4 µl of 10X enhancer containing Betaine (Epicentre). 5.3 µl of ddH_20, 1.0 µl of 10X BSA, 1.0 µl of primer (.00667 nmol), 1.0 µl template DNA (20-40 ng), and 1 unit of *Taq* polymerase (Promega) were added to the buffer solution to complete the reaction mixture. Primer sequences were obtained from the University of British Columbia (UBC) Biotechnology Laboratory **(**see Tsumura et al., 1996). All primers used in the final analyses were prepared by Life Technologies (Rockville, Maryland) based on UBC sequences. ISSR reactions were loaded in 96 well PCR plates while on ice and PCR was performed in a MJ Research (Watertown, MA) PTC-100 Thermocycler. The initial denaturation step was set to run for 1 minute at 94°C. Then 34 cycles of 45 seconds at 94°C, 30 seconds at 50°C, and 2:05 at 72°C were run. The final extension step was run for 5 minutes at 72°C. PCR

products were analyzed in 1.5% agarose gels and stained in an ethidium bromide solution on an orbital shaker.

Two samples from a single population were used for initial primer screening. Band sizes were estimated using 100 bp ladder (NEB, Beverly, MA). Loci were named based on

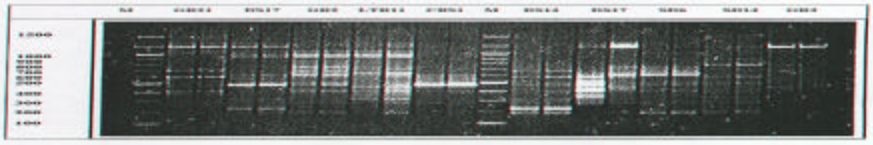
the primer used and estimated band size. Duplicate reactions were run for all ISSR analyses to determine the replicability of banding patterns.

All genetic analyses were run on both primers separately and together to determine the contribution of each to the combined genetic results.

Bands were scored based on presence or absence (See Figure 3 and Figure 4 for examples of gel photographs). Number of polymorphic loci, measures of the distribution of genetic diversity, Nei's genetic identity (*h*) (1973), and Shannon index of phenotypic diversity (*I*) (King and Schaal, 1989) were computed with PopGene32 (Yeh et al., 2000) assuming all loci were dominant and in Hardy-Weinberg equilibrium. An unbiased genetic identity matrix (Nei, 1978) using all populations of *A. oniciformis* and the single population of *A. mulfordae* was generated by PopGene32 and used to created a UPGMA dendrogram using NTSYSpc 2.02 (Rohlf, 1997).

Measures of population level genetic differentiation were determined using a hierarchical analysis of molecular variance (AMOVA; Excoffier et al, 1992) computed with Arlequin 1.1 (Schneider et al., 1997) which assumes these

Figure 4. Photo of DGK gel showing bands from mixed population using UBC#818. This gel was used to confirm band sizes across populations. M is a 100 base pair ladder.



data are haplotypic. For the estimation of genetic distances between populations, a pairwise population F_{st} matrix was generated by Arlequin 1.1 and an unbiased genetic distance matrix (Nei, 1978) was generated by PopGene32. Geographic distances were measured based on the latitude and longitude coordinates of the populations of *A. oniciformis*. Coordinates were obtained for four populations (DS, SD, LTR, and SB) using a Magellan (San Dimas, CA) Trailblazer XL commercial global positioning system (GPS) unit. Coordinates for the other populations were converted from Township, Range and Section (TRS) data obtained from Moseley and Popovich (1995) to latitude and longitude using TRS-data (http://WWW.esg.montana.edu/gl/ trs-data.html). Population coordinates were then imported as a point Shapefile into ArcView GIS 3.2 (Environmental Systems Research Institute, Inc., 1999) and geographic distances between populations were measured within a View. The population pairwise F_{st} matrix, Nei's unbiased genetic distance matrix, and geographic distance matrix were used in NTSYSpc 2.02 for the Mantel Tests to determine if population level genetic distances and geographic distance are correlated.

Correlation between the pairwise F_{st} and unbiased genetic distance matrices, between the pairwise F_{st} and unbiased genetic identity matrices, and between Nei diversity (h) and Shannon diversity (I) were conducted in SYSTAT for Windows ver. 6.0 (SPSS, Inc., 1996).

Estimation bias can lead to the overestimation of parameters by as much as 5% in the dominant marker data produced by RAPD and ISSR analysis (Lynch and Milligan, 1994). To reduce this bias, Lynch and Milligan (1994) proposed pruning any locus with a band frequency of higher than 1-(3/N), where N is the number of individuals sampled. This pruning procedure was implemented with the *A. oniciformis* data set prior to the genetic analyses.

Loci in Primer 818 and 841 for all populations of *A. oniciformis* and for the single population of *A. mulfordae* were tested for significant single population linkage disequilibria (Weir, 1979) and Ohta=s two locus analysis of population subdivision (1982, 1982a; both tests for linkage disequilibria) using PopGene32.

PAUP* for Windows ver. 4.0 beta 6 (Swofford, 2001) was used to assess the relationships among individuals of *A. oniciformis* and *A. mulfordae* using cladistic methodologies.

RESULTS

From an initial analysis of 100 UBC ISSR primers, the presence of multiple bands was found in 27 primers. Table 3 shows the primers that produced single bands in *A. oniciformis* and Table 4 shows the primers that produced multiple bands. From the subset of primers that produced multiple bands, reactions using two individuals from two populations were run to test for band replicability. Eight primers had bands that had a high degree of replicability. These eight primers were then run with a single individual from each of four populations with replicates. Two primers were then selected for the genetic analyses, UBC-818 and UBC-841. These two primers produced multiple, clear, and replicable bands that had a degree of heterogeneity across populations (Figures 3 and 4). A table of the raw data from *A. oniciformis* and *A. mulfordae* can be found in Appendix A. A list of the primer sequences and band sizes can be found in Table 5. In *A. oniciformis*, UBC-818 yielded 28 putative loci all of which were polymorphic. UBC-841 yielded 12 putative loci all of which were polymorphic (Table 6). Locus 841-775 was eliminated from the genetic analyses due to a significant linkage disequilibria existing between it and 841-475 (see linkage disequilibria results below).

Table 4. Primers yielding multiple bands in *A. amazonica*. Y = C or T; R = A or G; H = A, C or T; V = A, C or G.

Primer Name	Sequence
UBC 807	(AG)8T
UBC 810	(GA)8T
UBC 811	(GA)8C
UBC 812	(GA)8A
UBC 813	(CT)8T
UBC 814	(CT)8A
UBC 815	(CT)8G
UBC 816	(CT)8AT
UBC 817	(CT)8CA
UBC 818	(GA)8CG
UBC 819	(GA)8TG
UBC 820	(GT)8TC
UBC 821	(GT)8TG
UBC 822	(TC)8AG
UBC 823	(TC)8TG
UBC 824	(TC)8AC
UBC 825	(AC)8T
UBC 826	(AC)8G
UBC 827	(AC)8C
UBC 828	(TG)8A
UBC 829	(TG)8C
UBC 830	(TG)8G
UBC 831	(TG)8AA
UBC 832	(TG)8AG
UBC 833	(GAA)6
UBC 834	(AGA)6
UBC 835	(AG)8YC
UBC 836	(AG)8YA
UBC 837	(AG)8YG
UBC 838	(TC)8RA
UBC 839	(TC)8RG
UBC 840	(TC)8RC
UBC 841	(GAA)6RC
UBC 842	(GAA)6RG
UBC 843	(GAA)6RA
UBC 844	(CT)8RC
UBC 845	(CT)8RG
UBC 846	(CT)8RA
UBC 847	(CA)8RC
UBC 848	(CA)8RG
UBC 849	(TG)8YA
UBC 850	(TG)8YC
UBC 851	(TG)8YG
UBC 852	(TC)8RT
UBC 853	(TC)8RT
UBC 854	(TC)8RG
UBC 855	(AC)8YT
UBC 856	(AC)8YA
UBC 857	(AC)8YG
UBC 858	(GT)8RT
UBC 859	(GT)8RC
UBC 860	(TG)8RA
UBC 861	(ATG)6
UBC 862	(AGG)6
UBC 863	(ACA)6
UBC 864	(ACC)6
UBC 865	(ATC)6
UBC 866	(CTC)6
UBC 867	(GTC)6
UBC 868	(GAA)6YT
UBC 869	(GAA)6YC
UBC 870	(GAA)6YA
UBC 871	(GAA)6YG

Table 6. Genetic diversity measures for all sampled populations of *A. mexicanus*. Total number of loci, number of polymorphic loci, mean expected heterozygosity within a randomly mating subpopulation and mean expected heterozygosity within a randomly mating population with their respective standard deviations (s.d.), mean genetic diversity among subpopulations and mean number of individuals migrating between subpopulations per generation were calculated using PopGene32 (Yeh et al., 2000)

Primer	# of loci	# of polymorphic loci	H_S	s.d.	H_T	s.d.	G_{st}	Nm
B19	38	38						
B41	12	12	0.1859	0.05	0.1683	0.0226	0.1134	1.91
B19 & B41	50	50	0.1574	0.051	0.1898	0.0083	0.1705	1.21
			0.17	0.055	0.1807	0.0182	0.1185	1.91

Calculated allele frequencies and gene diversity statistics for *A. oniciformis* and *A. mulfordae* can be found in Appendix B.

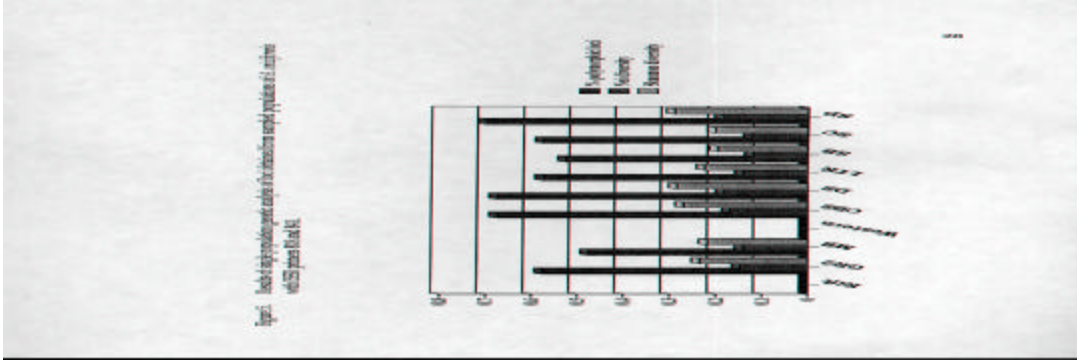
Twenty-three loci were present only in *A. oniciformis*, eighteen from UBC-818 (frequency = 0.0042-0.3675) and five from UBC-841 (frequency = 0.0042-0.0710).

In *Astragalus mulfordae*, UBC-818 yielded 11 putative loci all of which were polymorphic except 818-400. UBC-841 yielded 12 putative loci all of which were polymorphic. Six loci were present only in *A. mulfordae*, one from UBC-818 (frequency

= 0.0339) and five from UBC-841 (frequency = 0.0339-0.2697) . Locus 841-775 and 841-475 were not linked in *A. mulfordae*.

In *Astragalus oniciformis* and *A. mulfordae,* loci of band size lower than 400 were eliminated due to having an incomplete data set of these loci. Runs as long as 12 hours at 70 volts in the electrophoresis rigs are needed to resolve these bands. Many of the first gels were run for only 3 to 3.5 hours at 130 volts. The longer runs in the gel rigs were instituted so bands within 25 bp of each other would be further apart to make scoring easier.

The number of polymorphic loci within each population and their diversity indices varied depending upon whether the primers were analyzed separately or together. In a combined analysis (Table 7 and Figure 5), SD had the highest number of polymorphic loci, 28 (h = 0.1856, I = 0.2895). MB and SB were the most depauperate, with 19 and



polymorphic loci, respectively (MB: h = 0.1412, I = 0.2182; SB: h = 0.1189; I = 0.1949). The analysis of primer 818 (Table 8 and Figure 6) showed that DS had 23 polymorphic loci (h = 0.2339, I = 0.3596) and SD had 21 (h = 0.2043, I = 0.3145). LTR had the lowest, having 14 polymorphic loci (h = 0.1396, I = 0.2172). The analysis of primer 841 (Table 9 and Figure 7) showed that CBS and LTR both had 9 polymorphic loci (CBS: h = 0.1545, I = 0.2571; LTR: h = 0.1421, I = 0.239). DS,

GR2, and MB had the lowest number of polymorphic loci, 4 (DS: h = 0.061, I = 0.1058; GR2: h = 0.0982, I = 0.1512; MB: h = 0.061, I = 0.1058).

Nei diversity (h) and Shannon diversity (I) were highly correlated (r=0.9957, p=0). Unbiased Nei distance and genetic distance as estimated by pairwise F_{st} values were negatively correlated (r=-0.7751; p=0) and unbiased Nei identity and the pairwise F_{st} values were negatively correlated (r=-0.8275; p=0).

On an UPGMA dendrogram based on a Nei's (1978) unbiased genetic identity matrix, no populations with a separation of less than 25 km were grouped as most similar (unbiased genetic identity = 0.9691-0.9856). Two of the most distant populations were nearly identical, the rift population, MB, and the southwestern-most population, SD (unbiased genetic identity = 0.9902). *A. mulfordae* (SHS) was the most genetically different population sampled in this study (unbiased genetic identity = 0.9544; Figure 8). An

Table 9. Results of single population genetic analysis of loci from sampled populations of *A. [illegible]* with ISSR primer 841 using PopGene32 (Yeh et al., 2000).

	# of polymorphic loci 12 loci total	% polymorphic loci	Nei diversity h	Shannon index I
BIR				
EIR	3	0.3333	0.0983	0.1212
NIR	3	0.3333	0.061	0.1008
Western				
CRB	9	0.75	0.1535	0.2571
DS	4	0.3333	0.061	0.1058
ETR	9	0.75	0.1421	0.235
SR	5	0.4167	0.1006	0.1000
SC	8	0.6667	0.1195	0.2009
SD	7	0.5833	0.1418	0.2312

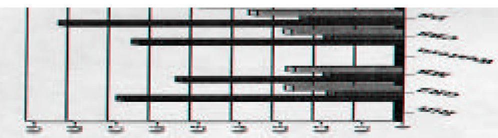
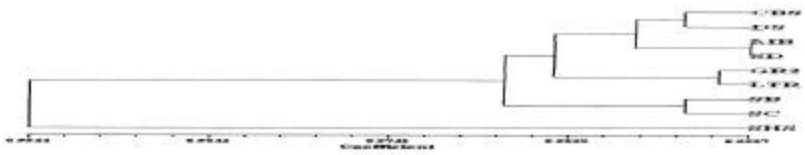


Figure 8. Unbiased genetic identity (Nei, 1978) UPGMA dendrogram generated with NTSYSpc (Rohlf, 1992). Population SHB is the only population of *Aegagropila* analyzed for this study; All others are sampled populations of *A. consimilis*.



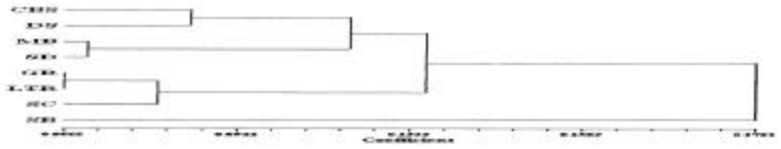
UPGMA dendrogram based on a Nei's (1978) unbiased genetic distance matrix showed the same relationships (Figure 9). A pairwise F_{st} UPGMA dendrogram was also generated with NTSYSpc (Rohlf, 1997; Figure 10).

AMOVA analyses of the combined 818 and 841 data (Table 10) found that 88.69 percent of the variation was significantly attributed to the variation within populations (p = 0) and that differentiation between the rift populations and the western populations was insignificant (p = 0.97) The results for the separate AMOVA analyses of Primers 818 and 841 have similar results (Table 11 and Table 12). These results also suggest that the rift populations and the western populations are not significantly differentiated.

The percent deviation from Hardy-Weinberg equilibrium due to population subdivision (G_{st}) and estimated gene flow between subpopulations per generation of sampled *A. oniciformis* populations (*Nm*) were nearly equal, whether the primers were analyzed combined or separately (G_{st}=0.113-0.1134, *Nm* = 3.91-3.93; Table 6).

The G_{st} values for the combined and separate analyses, are nearly identical to the **_st** values in the AMOVA analyses, between 0.112 and 0.118.

Figure 10. Pairwise F_{ST} UPGMA dendrogram generated with NTSYSpc (Rohlf, 1992). All populations are *A. oniciformis*



An additional AMOVA analysis was performed on the combined *A. oniciformis* data and *A. mulfordae* data, testing whether a significant

Table 11. AMOVA pairwise distance results from loci sampled from populations of *A. cruciformis* using ISSR primer #18. Two groups, the rift populations (CHE and PAB) the western populations (CHE, DIS, LHK, SB, SC, and SD) were tested in the genetic structure analysis. Loci analyzed with Arlequin (Schneider et al, 1997).

	d.f.	sum of squares	variance	% variation	Φ statistics	P
Between Groups	1	4.3	-0.15	-4.4	Φ_{SC}= -.044	1
Among Populations/Groups	6	88.87	0.54	15.56	Φ_{ST}= 0.145	0
Within Populations	113	344.13	3.07	88.84	Φ_{CT}= 0.112	0
Total	119	415.3	3.46			

Table 15. AMOVA pairwise distance results from loci sampled from populations of *A. crassiformis* using ISSR primer 841. Two groups, the rift populations (CHP and NHP) the western populations (CHS, DS, LHK, SB, SC, and SD) were tested in the genetic structure analysis. Test analyzed with Arlequin (Schneider et al. 1997).

	d.f.	sum of squares	variance	% variation	st statistics	P
Between Groups	1	3.47	-0.007	-0.62	Φ_{CT}= -.006	0.51
Among Populations/Groups	5	17.8	0.13	12.41	Φ_{SC}= 0.123	0
Within Populations	113	106.5	0.95	88.2	Φ_{ST}= 0.118	0
Total	119	127.37	1.08			

amount of variation was explained by groups of all *A. oniciformis* populations and grouping the single *A. mulfordae* population (Table 13).

A weakly significant (p = 0.1) 17.34 percent of the variation was explained by this grouping.

UPGMA dendrogram of the unbiased genetic identity (Nei, 1978; Table 14 and Figure 8) showed that populations MB and SD were nearly identical (unbiased genetic identity = 0.9925). This result is significant since MB and SD are 67 km apart, the only further distant populations are between the rift populations, GR and MB, and CBS (82 and 80 km, respectively), and neither are paired as similar.

A Mantel Test using the geographic distance matrix and Nei=s (1978) unbiased genetic distance matrix found that they weakly correlated (t = -0.34809; p = 0.0780; Table 15 and Figure 11). Another Mantel Test using the geographic distance matrix and pairwise F_{st} genetic distance matrix found that they are not significantly correlated (t = -0.27905; p = 0.1135; Table 16 and Figure 12). Genetic distances among populations of *A. oniciformis* are perhaps weakly correlated with respect to geographic distance.

Significant linkage disequilibria was found to occur between locus 841-775 and locus 841-475 (Table 17 and Table 18). Weir=s single population linkage disequilibria

Table 14. Unbiased genetic identity (Nei, 1978) and geographic distance. Geographic distance (km) is below the diagonal. Unbiased genetic identity is above. Genetic identity was estimated with PopGene32 (Yeh et al., 2000) by calculating the unbiased genetic identity of all loci from ISSR primers 818 and 841 from all *A. californica* populations.

	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]		[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	

test found that most of the populations had a significant linkage disequilibria between these

two loci (corr. = 1; Chi-square

Table 15. Unbiased genetic distance (Nei, 1978) and geographic distance. Geographic distance (km) is below the diagonal. Unbiased genetic distance is above. Genetic distance was estimated with PopGene32 (Yeh et al., 2000) by calculating the unbiased genetic distance of all loci from ISSR primers 818 and 841 from all 4 *[illegible]* populations.

	CBS	DS	GB2	LTB	MB	SB	SC	SD
CBS		0.0121	0.0188	0.0297	0.0163	0.0301	0.0206	0.0304
DS	37.3		0.0178	0.0262	0.0098	0.0225	0.0252	0.0151
GB2	82.78	55.95		0.0297	0.0178	0.0229	0.0167	0.0229
LTB	98.5	41.16	33.74		0.0151	0.0314	0.019	0.0188
MB	80.25	55.55	5.16	34.87		0.0252	0.0179	0.0075
SB	52.32	33.78	33.14	8.09	35.46		0.0118	0.0081
SC	44.16	8.54	50.11	35.50	50.11	27.65		0.0236
SD	16.45	59.82	79.35	47.31	67.05	45.69	44.2	

Table 16. Genetic distance and geographic distance. Geographic distance (km) is below the diagonal. Genetic distance is above. Genetic distance was estimated with Arlequin (Schneider et al., 1997) by calculating the pairwise F_{st} of all loci from ISSR primers 818 and 841 from all *A. [illegible]* populations.

	CHI?	DS?	CR2?	UTR?	MB?	SB?	RC?	MI?
CHI?		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
DS?	[illegible]		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
CR2?	[illegible]	[illegible]		[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
UTR?	[illegible]	[illegible]	[illegible]		[illegible]	[illegible]	[illegible]	[illegible]
MB?	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]	[illegible]	[illegible]
SB?	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]	[illegible]
RC?	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]		[illegible]
MI?	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	

Figure 12. Scatterplot of geographic distance versus pairwise F_{ST} values generated with NTSYSpc (Rohlf, 1992) for *A. orbiculoides*.

Matrix correlation: r = -0.37905;
Approximate Mantel t-test: t = -1.2082;
Probability of random F_{ST} values> observed F_{ST} value: p = 0.1135

Table 17. Single population linkage disequilibria (Weir, 1979). Loci are from analysis of *A. orbiculoides* with ISSR primer #41 using PopGene32 (Yeh et al., 2000).

Pop.	Locus 1 (Allele)	Locus 2 (Allele)	LD	Correlation	Lewontin	Chi-square	P
1	376 [0]	405 [1]	0.13	0.6124	0.70	5.63	0.018
2	376 [0]	405 [1]	0.13	0.6124	0.68	5.63	0.018
3	376 [0]	405 [1]	0.13	0.6124	0.68	5.63	0.018
4	376 [0]	405 [1]	0.134	0.728	0.75	8.64	0.018
5	376 [0]	405 [1]	0.134	1	1	17	0.0001
6	376 [0]	405 [1]	0.134	1	0.75	17	0.0001
7	376 [0]	405 [1]	0.134	1	0.68	17	0.0001
8	376 [0]	405 [1]	0.134	1	1	17	0.0001
9	376 [0]	405 [1]	0.134	1	1	17	0.0001
10	376 [0]	405 [1]	0.133	1	0.71	17	0.0001
11	376 [0]	405 [1]	0.133	1	0.64	17	0.0001
12	376 [0]	405 [1]	0.133	1	1	17	0.0001
13	376 [0]	405 [1]	0.133	1	1	17	0.0001
14	376 [0]	405 [1]	0.116	1	0.64	17	0.0001
15	376 [0]	405 [1]	0.134	1	1	17	0.0001
16	376 [0]	405 [1]	0.134	1	0.75	17	0.0001
17	376 [0]	405 [1]	0.134	1	0.75	17	0.0001
18	376 [0]	405 [1]	0.134	1	1	17	0.0001
19	376 [0]	405 [1]	0.134	1	0.889	17	0.0001
20	376 [0]	405 [1]	0.106	1	0.67	17	0.0001
21	376 [0]	405 [1]	0.106	1	1	17	0.0001
22	376 [0]	405 [1]	0.106	1	1	17	0.0001
23	376 [0]	405 [1]	0.208	1	0.32	17	0.0001
24	376 [0]	405 [1]	0.208	1	0.286	17	0.0001
25	376 [0]	405 [1]	0.132	1	1	17	0.0001
26	376 [0]	405 [1]	0.116	1	1	17	0.0001
27	376 [0]	405 [1]	0.116	1	0.64	17	0.0001
28	376 [0]	405 [1]	0.116	1	0.64	17	0.0001

Table 18. Ohta's two-locus analysis of population subdivision (1982, 1982a) to test for significant linkage disequilibrium among multiple populations. Loci are from an analysis of *A. [illegible]* with ISSR primer 841 using PopGene32 (Yeh et al., 2000).

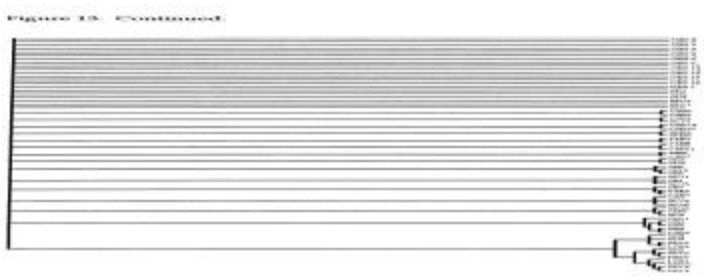
Locus A	Locus B	$(D_{IT})^2$	$(D_{IS})^2$	$(D'_{IS})^2$	$(D_{ST})^2$	$(D'_{ST})^2$
[illegible]	[illegible]	0.257	0.148	0.075	0.087	0.182

test, p=0.001). CBS still had significant linkage disequilibria between these two loci, but it was not as strong as in the other populations (corr.=0.6123; Chi-square test, p=0.018). Ohta's two-locus analysis of population subdivision (1982, 1982a) showed significant

linkage between these two loci($\{D\text{-}ST\}^2 = 0.183$). Based on these results, locus 841-775 was deleted from all genetic analyses.

For a locus to be pruned according to Lynch and Milligan (1994) in the *A. oniciformis* ISSR data (N=120), the frequency of the band had to be 0.975 or higher. Since no locus was present at a frequency of higher than 0.88, the Lynch and Mulligan pruning procedure was not implemented.

In PAUP*, an initial heuristic search of the combined 818 and 841 data sets of both *A. oniciformis* and *A. mulfordae* of 100 random addition sequences with TBR branch swapping stopped during the first addition sequence after the tree buffer filled to capacity. A set of 10,000 most parsimonious trees of length 397 were recovered. To find if shorter trees existed, another heuristic search of 10,000 random addition sequences was performed. Only 25 trees of length 397 or longer were held at each step. The analysis was stopped at step 5229 when trees no shorter than 394 were found. A set of 86,959 most parsimonious trees of length 394 were recovered (RI = 0.5956, CI excl. uninf. = 0.1010). Figure 13 is a strict consensus of the length 394 trees.



DISCUSSION

The nearly identical **_st** values from the AMOVA analysis and the G_{st} values from the PopGene32 analysis is evidence that potential problems arising from the violation of certain assumptions of the software did not occur. ISSR studies generally use the same software algorithms and methodologies used in this study, but very few test their ISSR data to determine the extent to which the potential violations bias their results. The analyses used in PopGene32 assumes that all data are in Hardy-Weinberg equilibrium. Since no codominant ISSR locus was found and codominant data do not exists for *A. oniciformis*, the assumption that the data meets Hardy-Weinberg expectations could not be tested. The

AMOVA procedure in Arlequin assumes the data are codominant and haplotypic. ISSR data could cause bias in parameter estimation since ISSR markers are dominant and not haplotypic. In addition to the identical **_st** values and G_{st} values, the topology of the UPGMA dendrograms of the pairwise *Fst* and Nei=s unbiased genetic distance were nearly identical, differing only in branch lengths and the grouping of the SB and SC populations (Figure 8 and 10). The significant correlation between the pairwise *Fst* values (calculated by NTSYSpc) and the Nei=s unbiased genetic distance values (calculated by PopGene32) is also

encouraging evidence. The two different ISSR primers produced, in separate and combined analyses, nearly identical results. These results were verified in the AMOVA analyses. In addition, parameter estimation bias can be reduced by the Lynch and Milligan test, and since no bands in these data were present at a high enough frequency to be pruned, bias in the *A. oniciformis* data is low. The data from populations of *A. oniciformis* demonstrate the robustness of ISSR markers.

Studies of other species of *Astragalus* using genetic methods such as isozymes, AFLPs, or RAPDs have yielded similar results as ISSR markers have in *A. oniciformis* and

A. mulfordae. In an isozyme study among populations of various species of annual *Astragalus*, Liston (1992) found that Nei's genetic identity did not fall below 0.961. The tight range of genetic identities 0.97-0.99 found in *A. oniciformis* with ISSR markers is on the high end of the range of values reported by Liston (1992).

It can be difficult to compare the ISSR results obtained in this study with similar studies utilizing other methodologies. Karron et al. (1988) compared the genetic structure of widespread species of *Astragalus* with endemic species having narrow distributions using allozymes. Levels of polymorphism were assayed based on 25 individuals from three populations of each of four species. Twelve loci were sampled in each population. Two (17%) were polymorphic in *A. osterhouti*, a species with four known

populations in Colorado. The highest number of polymorphic loci, four (33%), was found in *A. linifolius*, a species restricted to three populations in Colorado, and *A. pectinatus*, a widespread species. *A. pattersoni* had an intermediate level of polymorphic loci, three (25%). In contrast, the single population of *A. mulfordae* had 96% polymorphic loci, and all loci were polymorphic in *A. oniciformis*. Since allozymes and ISSRs produce different levels of polymorphic loci (Esselman et al., 1999), a direct comparison between *A. oniciformis* and the species studied by Karron et al. (1988) is difficult.

The genetic identity of 0.95 between *A. mulfordae* and *A. oniciformis* is not unusual. Liston (1992) found genetic identities between several annual species of *Astragalus* ranged from 0.404 to 0.937. A genetic identity of 0.937 was found between *A. breweri*, a species native to serpentine outcrops in the Coast Range of California and *A. tener* var. *titi*, which is found in a single population on the Monterey Peninsula of California (Liston, 1992). Like *A. mulfordae* and *A. oniciformis*, these two annual species are not sympatric. The genetic identity obtained in this study between *A. mulfordae* and *A. oniciformis* is likely to be inaccurate, since only one population was sampled. A more thorough sampling of populations of *A. mulfordae* will potentially provide additional loci in *A. mulfordae* that are currently only present in *A. oniciformis*, as well as additional loci unique to *A. mulfordae*.

The cladistic analysis of the *A. mulfordae* and *A. oniciformis* data, resulted in a highly unresolved strict consensus tree. In addition, most individuals of *A. mulfordae* were unresolved in a large polytomy with a majority of the *A. oniciformis* individuals. Only one band clearly differentiated *A. mulfordae* from *A. oniciformis*. Band 818-400 was the only band in this study that was not polymorphic in *A. mulfordae* and it was present at an average,low frequency of 0.227 in *A. oniciformis*. The low level of differentiation

contributed to the lack of resolution in the cladistic analysis and the relatively high genetic identity between the species.

Geologic features and habitat restrictions have been documented as instrumental in increasing population differentiation in species with limited distributions (Travis et al., 1996). In *Astragalus cremnophylax*, a species native to Kaibab Limestone outcrops on the North Rim and South Rim of the Grand Canyon, genetic differentiation overall among the populations is high , _ (an equivalent of F_{st}) = 0.44, (Travis et al., 1996) compared to *A. oniciformis Gst*=0.113. Gene flow (*Nm*) is limited for *A. cremnophylax*, between 0.2 and 0.4 migrants per generation. Gene flow has been proposed only to occur through pollinators, since geographic barriers (The Grand Canyon) and habitat barriers (16 km of dense vegetation) prevent seed dispersal. The population sizes of *A. cremnophylax* ranged from 2 to 970 individuals, which makes this species extremely vulnerable to fluctuations in climate and habitat

disturbance (Travis et al., 1996). *A. oniciformis* has a much wider, continuous distribution, over 80 km, larger population sizes 10 to >10,000 individuals, and higher estimates of gene flow (*Nm* = 3.91 to 3.93). The lack of genetic differentiation among populations, especially

when compared to *A. cremnophylax*, is also evidence that a very high gene flow exists throughout the range of this species.

A wide range of G_{st} and F_{st} values have been obtained in studies of species of *Astragalus* (Table 19). Liston (1992) found that within annual species, G_{st} values ranged from 0 to 0.725. The highest values were found in *A. pauperculus* (0.775) and *A. clarianus* (0.331), two species with narrow distributions in cismontane California. The widespread species had G_{st} values between 0 and 0.254. The highest F_{st} values in Karron et al. (1988) was found in *A. osterhouti* (F_{st} = 0.14), which in 1988 had a total of 1500 individuals restricted to three populations. Comparatively, *A. pectinatus* (F_{st} = 0.02 and 0.05) and *A. pattersoni* (F_{st} = 0.01) are widespread species with lower levels of genetic differentiation than observed in this study (Karron et al., 1988). A__ = 0.44, found in A. *cremnophylax* (Travis et al., 1996), is additional support that in *Astragalus*, genetic differentiation and possibly speciation can occur when population size decreases, gene flow decreases and genetic differentiation among populations increases in endemic species with narrow distributions. Although *Astragalus oniciformis* has a relatively narrow distribution, its large

Table 19. Mean population F_{ST}s and populations sizes from studies of species of *Astragalus*.

Species	Mean Population Differentiation	Population Size	Distribution	Citation
Astragalus	0.1 (Gst)	10-200	narrow	Linhart, 1992; Linhart, 1991;
Astragalus	0.1 (Gst)	< 35	narrow	Linhart, 1992; Linhart, 1991;
Astragalus	0.065 (Gst)	35-50	narrow	Linhart, 1992; Linhart, 1991;
Astragalus	0.060 (Gst)	3,000	narrow	Barrow et al, 1988;
Astragalus	0.055 (Gst)	40-350	narrow	Linhart, 1992; Linhart, 1990;
Astragalus	0.058 (Gst)	100-1000	narrow	Linhart, 1992; Linhart,
Astragalus	0.115 (Gst)	100-10,000	narrow	Meagher & Pigeon, 1995;
Astragalus	0.13 (Gst)	1,000	narrow	Barrow et al, 1988;
Astragalus	0.33 (Gst)	< 100	narrow	Linhart, 1992; Linhart, 1990;
Astragalus	0.34 (Nei)	5,000	narrow	Travis et al, 1996;
Astragalus	0.726 (Gst)	35	narrow	Linhart, 1992; Linhart, 1991;
Astragalus	0 (Gst)	?	widespread	Linhart, 1992; Linhart, 1991;
Astragalus	0 (Gst)	?	widespread	Linhart, 1992; Linhart, 1991;
Astragalus	0 (Gst)	?	widespread	Linhart, 1992; Linhart, 1991;
Astragalus	0.05 (Gst)	?	widespread	Barrow et al, 1988;
Astragalus	0.05 (Gst)	?	widespread	Barrow et al, 1988;
Astragalus	0.311 (Gst)	?	widespread	Linhart, 1992; Linhart, 1991;
Astragalus	0.251 (Gst)	?	widespread	Linhart, 1992; Linhart, 1991;

population sizes, numerous occurrences, and high gene flow among populations has resulted in a low potential for genetic differentiation.

A number of factors about the characteristics of the sampled populations could have had an impact on the genetic analyses performed in this study.

The type locality, located near the eastern city limits of Picabo, was not sampled due to the depauperate condition of the populations. SC was the nearest population to the type locality of sufficient size to be sampled. The observed habitat fragmentation at the type locality is likely to have some effect on the genetic differentiation of that population. SC was located in a small undisturbed patch of *Artemisia* between several large private farms. Even though habitat fragmentation in part does not seem to have affected this species genetically

(see discussion of populations MB and GR2 below), habitat fragmentation and low population size has the potential to significantly affect the levels of genetic differentiation among populations (Travis et al., 1996). If decreases population sizes and habitat fragmentation continues to occur within the northern range of this species, mainly in the populations around Picabo and Silver Creek, the combination of the two may lead to genetic differentiation among these populations and populations throughout the range of the species.

One historical population (SD) was relocated shortly before the collection survey for this study, and because it represents the southernmost known population of this species, it was sampled. This population is the only one in this study that has not been adequately investigated.

The rift populations, GR2 and MB, located within a 5 mile radius of Mule Butte, was sampled from the four disjunct eastern populations of this species, separated from all other populations by the eight mile wide, inhospitable Minidoka Flow (Moseley and Popovich, 1995). The lack of genetic differentiation between these two populations and the western populations provides evidence that in *A. oniciformis*, either the rift populations are

the result of 2 or more recent dispersal events or 3600 years of separation has not caused significant genetic differentiation between the rift and western populations. Two or more dispersal events are possible since MB and GR have different levels of polymorphic loci, are not grouped as being similar in the UPGMA dendrograms, and have a genetic identity of 0.9824, a value in the middle of the range for this species. Gene flow across this inhospitable boundary has not been completely ruled out since the life histories of the pollinators of *A. oniciformis* have not been studied (Popovich, personal communication, 1999).

The lack of genetic differentiation among populations and the high level of gene flow within the range of *A. oniciformis* indicates that current

threats to this species, plant community changes due to changing fire patterns, habitat alteration due to livestock grazing, and habitat loss due to agricultural development (Moseley and Popovich, 1995) have not effected the genetic diversity of this species. Genetic differentiation has not occurred despite these disturbances because of the high gene flow and the numerous, large populations characteristic of *A. oniciformis*. In addition, the seed bank for *A. oniciformis* can be potentially large (Pyke, personal communication, 2001), so if genetic differentiation were to occur, it could be several generations before genetic drift is

detectable. Conserving the numerous, large populations throughout the range of this species would be one strategy that would help preserve the high gene flow among populations.

The populations located near Picabo (see Figure 1) near the type locality and the populations along Silver Creek should not be selected as a seed source for habitat restoration or enhancement projects for other populations throughout the range of *A. oniciformis*. The low levels of polymorphism, low population sizes, and the higher potential for future genetic differentiation make these populations poor candidates. Populations within the continuous central and western range of this species are the best candidates for restoration and enhancement efforts.

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